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14. ABSTRACT

Two separate experiments where used to explore the effects of RTA 408 and metformin in mitigating the effects of radiation. In our first experiment we used a combined injury/simultaneous radiation model whereby a 2X2 cm wound was created on the abdomen of the rat with immediate radiation of 20Gy being delivered. They were then followed out 6 weeks and photos were taken weekly to determine if the study drugs where having an effect. No significant effects were found by administering RTA-408 or metformin. A trend towards faster wound healing was observed.

The second experiment was designed to look at the delayed effects of radiation. Animals were subjected to 40 Gy of radiation in 4 fractions of 10 gy over the course of a week. During this radiation they received DMSO (vehicle control) or RTA408. The animals were allowed to go through acute healing from the radiation and at 6 weeks they were challenged with a surgical insult of a rotational flap designed on the abdomen. The heartiness of the flap was measured by degree of necrosis after the animals were sacrificed 1 week postop. Findings confirmed RTA-408 when delivered during radiation resulted in significant improvement in flap survival. This is consistent with the conclusion that the benefit of RTA408 is less relevant in the acute wound healing setting but affects mechanisms relevant to resolution of radiation damage and, potentially chronic inflammation.

15. SUBJECT TERMS

Radiation, Acute Injury, Fibrosis, Thiol-Modifying Agent, RTA 408, Metformin

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Introduction:

Damage to normal tissues by accidental or intentional radiation exposure is a pervasive threat to military personnel and civilians alike. Dispersal of radioactive materials may occur as a consequence of nuclear reactor incidents (e.g. Fukushima) or following detonation of explosive devices laced with radioactive materials (e.g. 'dirty bomb'). The term 'combined injury' describes the simultaneous occurrence of radiation-associated damage and mechanical trauma in these settings. Acute and chronic radiation tissue damage have been extensively studied in the context of radiation therapy of cancer in which the therapeutic benefits of this treatment modality are curtailed by toxicity, both acute and delayed. This clinical experience largely informs our understanding of potential sequelae of non-medical radiation exposure. Compromised function due to chronic inflammation and progressive fibrosis affecting tissues within the radiation field have emerged as pervasive complications of radiation exposure affecting many patients for the rest of their lives and frequently causing complications during post-treatment restorative surgical procedures. This experimental design of this award was designed to assess the potential of a cytoprotective agent (RTA408) to ameliorate radiation toxicity to normal tissues and to preserve tissue function. RTA408 is a synthetic triterpenoid currently based on the CDDO structure with antioxidant and anti-inflammatory properties[1,2]. It belongs to a class of agents which alkylate cysteines including parthenolide which, like CDDO-derived compounds, has radioprotective properties[3]. RTA408 has recently been shown to radioprotect rodent skin against acute radiation injury[4]. It is in clinical development for other applications.

The overall goal of the work described below was to define whether radioprotection of irradiated rat abdominal skin by RTA408 benefits wound healing in the settings of (i) combined injury (radiation and wounding applied concurrently) or (ii) delayed surgical challenge of pre-irradiated skin. In the latter model preirradiated abdominal skin was subjected to flap surgery 6 weeks after irradiation and, as shown by us previously, pre-irradiation induces extensive flap necrosis at the distal end of the skin flap[5]. In all experiments irradiation was performed using external beam radiation (XRT) sparing internal organs.

Keywords:

Radiation, Acute Injury, Fibrosis, Thiol-Modifying Agent, RTA 408, Metformin

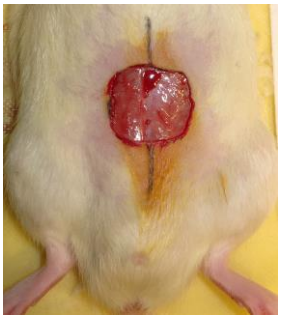
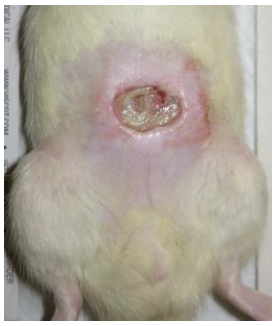
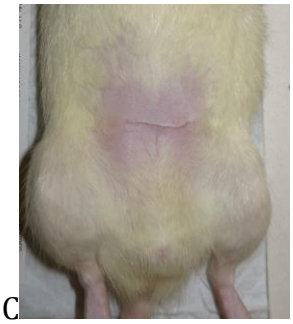
Accomplishments:

During the first 11 months of support through this award we have made significant progress. We have completed experiments described in Sections 1.1-4. of the Sow/Gantt chart (attached in the Appendix). Specifically, we have completed efficacy studies using RTA408 in both, the early combined injury model and the delayed flap model. Wound closure and surgical outcomes were documented by photographic imaging and quantitative image analyses performed to quantify the effects of RTA408 in the two settings. Tissue samples were secured in these experiments for ongoing morphological and molecular studies. In select experiments, we have also included a second agent (metformin) with similar cytoprotective properties. This was based on evidence in the literature that metformin and CDDO variants have significant overlap in biological effects and targets ranging from altering mitochondrial function and redox states of cells and organelles to NF-kB inhibition[6-8]. Finally, we have initiated analysis of fibrotic changes in the delayed surgery model.

Combined injury/XRT model. We first performed dose-finding experiments to identify the optimal radiation dose range to observe beneficial drug effects in the combined injury model. Briefly, the experimental protocol consisted of creating an abdominal wounding (2x2 cm) at the time of radiation followed by 60 days of monitoring during which wound closure was documented by serial photographs and biopsies taken at the conclusion of the experiments. Once we determined the appropriate radiation dose (20 Gy single dose) which caused significant impairment of skin appearance and function while significantly delaying wound closure we tested the effects of the experimental compounds. Of note, in one cohort approximately 50% of the animal died during anesthesia for reasons unknown but unrelated to drug treatment and potentially related to anesthesia complications. This cohort of animals was excluded form further analysis. Based on prior experience with RTA408 and metformin in mice the experimental groups were designed as follows:

i) Radiation/RTA408 (6 mg/kg) administered on days 1,2,3,4,5 post-radiation;
ii) Radiation/DMSO (vehicle control administered on days 1,2,3,4,5 post-radiation);
iii) Radiation/metformin (2.5mg/kg/day); added to drinking water for 6 weeks post-radiation);
iv) No radiation/DMSO (vehicle control administered on days 1,2,3,4,5 post-radiation).

Figure1: A) Initial wound at day zero. B) Wound appearance of irradiated (20Gy) and (C)control non-irradiated animals at postop day 14.

A  B  C 

Of note RTA408 was used at a dose previously determined to provide radioprotection to the gastrointestinal system of mice (Alexeev et al.,14).

The extent of wound closure over time was photographically documented on days 0, 3, 7 post-irradiation, and every week following that (representative images shown Fig. 1). Wound size was digitally analyzed to measure both total area at each time point as well as the length of the wound at its midpoint. The results of these analyses are shown in Figures 2 and 3.

Figure 2: Changes in wound area over time in control and RTA408-teated animals.

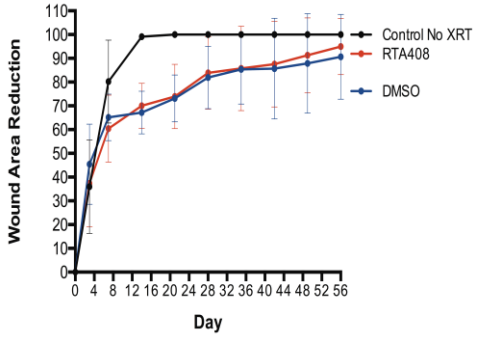
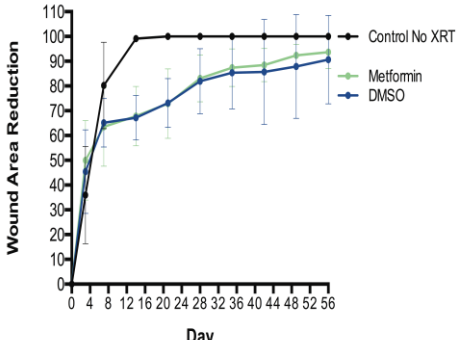
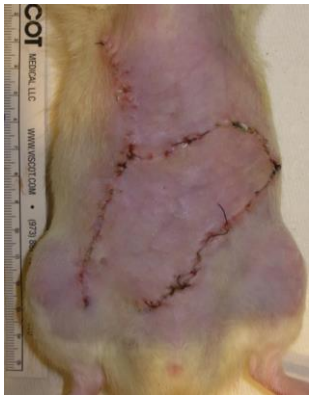
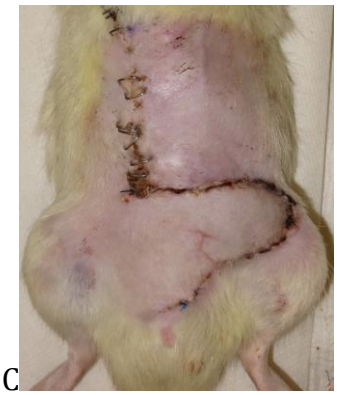


Figure 2: Changes in wound area over time in control and metformin-treated animals.



These experiments revealed marginally accelerated wound closure in drug-treated animals in some experiments with considerable variation between experiments. For example, in the third cohort, 6/8 of the RTA-treated animals exhibited complete healing during the study period (with an average healing time of 44 days), compared to 3/7 of the DMSO-treated animals (with an average healing time of 54 days). The 1st and 2nd cohort of experiments did not demonstrate this same promising response. Tissue specimens from this experiment were collected for analysis of collagen deposition, vascular density, and mRNA expression of mediators of chronic inflammation and fibrosis.

Figure 4: Induction of necrosis in skin flaps exposed to ionizing radiation. (A) Skin flap appearance 7 d after surgery in a control animal (no radiation); (B) Necrosis of the distal skin flap in a rat that received to 40Gy to the abdominal skin 60 d prior to surgery; (C) No necrosis of flap in radiated rats treated with RTA-408.

A  B  C 

Delayed injury/surgical challenge model. In this set of experiments rat abdominal skin was first exposed to radiation and 6 weeks later subjected to rotational flap surgery (Fig. 4) mimicking the clinical setting of post-treatment intervention in HNSCC. A total of 60 rats where used in these experiments. Radiation consisted of total dose of 40 Gy to the abdominal skin with limited dose to the gut (<10 Gy total) in 4 fractions delivered over 8 days. After 6 weeks of observation the animals were subjected to the axial rotational flap procedure. Follow-up was performed by daily photographic documentation and animals were sacrificed 7 days after surgery (~7 weeks after radiation). The primary 'clinical' endpoint of this study was the extent of flap necrosis (area of necrosis / total area of flap). The experimental groups consisted of:

Primary experiment

i) Radiation/RTA408 administered on days1,3,5,8 and 10, - N = 16
ii) Radiation/DMSO (vehicle control for RTA408) N = 12
iii) No radiation/DMSO control N = 13

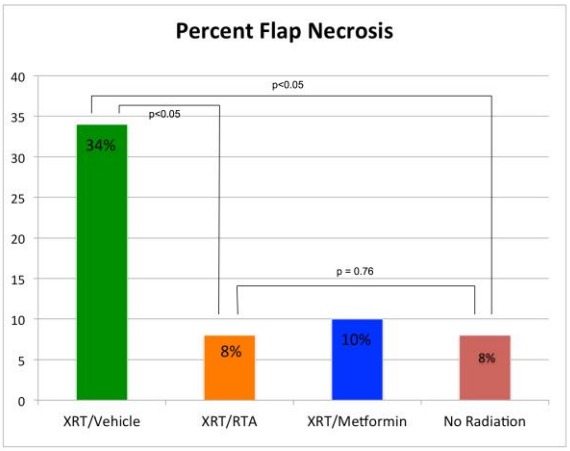
Secondary experiment

iv)Radiation/metformin administered (2.5mg/kg/day); added to drinking water for 6 weeks post-radiation) N = 6
v) Radiation/vehicle control for metformin N = 6

The extent of necrosis was statistically significantly reduced in animals receiving RTA408. Metformin trended towards a reduction in necrosis, but only one cohort of 6 animals received daily dose of metformin. As summarized in Fig. 5, in irradiated, vehicle-treated animals the distal third (34%) of the flaps was necrotic whereas animals receiving vehicle control and no radiation exhibited 8% necrosis. RTA408 treatment reduced the extent of necrosis to control levels in non-irradiated rats (approximately 8%,

and metformin treatment reduced flap necrosis to a lesser extent (10%) and was not powered (n=6) appropriately to run statistical analysis. The effect of RTA408 was statistically significant ($p<0.05$) As determined by ANOVA and post hoc Tukey test the RTA 408 effect on necrosis reduction was significant at $p=0.02$ whereas the effect of metformin was not statistically significant.

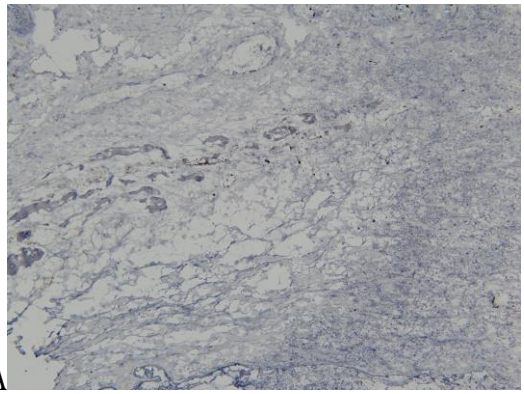
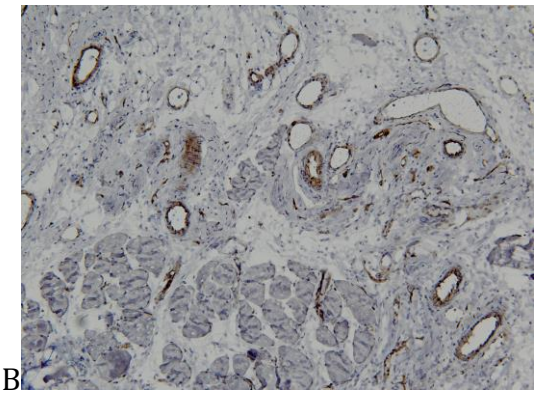
Figure 5: Percent necrosis in RTA408/metformin treatment in previously irradiated animals subjected to flap surgery.



Based on these results we conclude that RTA408 provides significant protection to full thickness pre-irradiated, surgically challenged skin. This effect is highly relevant to the clinical setting as RTA408 and related compounds may have utility in preserving tissue function and 'operability' for procedures performed post radiation treatment. Metformin also decreased the extent of postoperative necrosis but this effect was not statistically significant and awaits further testing as the number of animals in the treatment group was smaller (n=6) than in the other experimental vs (n=10).

We have begun the analysis of molecular and histological correlates of increased viability in RTA408-treated skin. A portion of samples have been processed for CD31 staining (marker of endothelial cells; Fig. 6) revealing a significant difference in vascular density in flaps that have received RTA408 compared to the vehicle (DMSO) control group. This is consistent with the clinical improvement in flap survival and suggest that preservation of blood supply might by one mechanism underlying the beneficial effects of RTA408 in this setting. In future work we will validate this observation,

Figure 6: Representative images of skin sections stained with CD 31 antibody for vessel identification. (A) XRT/ vehicle control and (B) RTA408-treated animals.

A  B 

Currently we are analyzing changes in the extracellular matrix structure (dermal thickness and collagen composition), and mRNA expression levels of markers of chronic inflammation and fibrosis. Our next report will have the analysis of the frozen and embedded specimens collected during this past 12 months.

Impact: Strategies to mitigate the effects of radiation exposure are limited and yet critical to reducing the impact of radiation exposure on acute and long-term morbidity. The results described above demonstrate a significant effect on mitigating the delayed effects of radiation with an endpoint that is clinically relevant to the clinical setting, i.e. surgical intervention in chronically damaged irradiated tissues. By contrast, we observed marginal benefit of the agents investigated in the combined injury model in which radiation and wounding are administered concurrently. This is consistent with the conclusion that the benefit of RTA408 is less relevant in the acute wound healing setting but affects mechanisms relevant to resolution of radiation damage and, potentially chronic inflammation. In conclusion we have identified an agent, which promises to improve long-term tissue integrity after radiation injury in military and civilian personnel in the event of radiation exposure as well as veterans undergoing radiation therapy for oncologic treatment.

Changes/Problems:

Combined injury/XRT model – The original proposal we planned to use 40 gy in a single dose. Prior to starting this experiment we embarked on titrating the radiation dose (20, 30 and 40 gy) to determine if a lower radiation dose would result in a clinically significant poor wound healing compared to controls. Measurable and reproducible effects were found at a single dose of 20 Gy without animal mortality.

The 6mg/kg dose of RTA408 was previously worked out in prior studies, but the duration of therapy to determine if a therapeutic effect can be identified in the combined injury model. We propose future study giving RTA 408 for the duration of the 6 weeks to see if healing is improved. In this proposal we were focused on mitigating the effects of radiation, thus the reason we chose a short interval of 5 days to deliver the drug around the time of the injury and radiation.

Delayed flap model after radiation – Model has been used in our lab for 4 years and well established. No changes or modifications during this experiment.

Products: No new products were developed. Metformin is a well-tolerated agent approved by the FDA for the treatment of type-II diabetes. RTA408 is currently under active clinical development for several indications including cancer therapy in combination with immunotherapy.

Participants and Other Collaborating Organizations:

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Project role:	PI
Contribution to Project	Design, experimental support, analysis
Funding Support	Clinical and DOD grant

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Project role:	Co-PI
Contribution to Project	Design, experimental support, analysis
Funding Support	DOD grant and other grant support

Name:	Al Linnenbach
Project role:	Lab Manager and Research technician
Contribution to Project	Experimental support, analysis
Funding Support	Clinical and DOD grant

Name:	Jonathan Bornstein
Project role:	Medical Student/Research assistant
Contribution to Project	Experimental support
Funding Support	Departmental Funds

Name:	Michelle Chen
Project role:	Medical Student/ Research assistant
Contribution to Project	Experimental support
Funding Support	Departmental funds

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Arresting inflammatory and fibrotic pathways in radiation induced injury using small molecule thiol modifying agensts

Thomas Jefferson University

Project Lead: Adam Luginbuhl
Project Start Date: ~6/1/2015
Display Week: 1

WBS	Task	Lead
0	**ACURO approval**	**Luginbuhl**
1	**Admininstratin of RTA408 and XRT of rats**	Luginbuhl
1.1	Order 80 Sprague Dawley Rats.	[Name]
1.2	Weigh out RTA in aliquots	
1.3	Injection of RTA or Vehicle control on day of radiation until 3 days post radiation	
1.4	Administration of 40 Gy radiation to animals	
2	**Secondary surgical event**	Luginbuhl
2.1	Creation of axial rotational flaps 60 days s/p radiation	
2.2	Photo documentation of flaps as they mature for 7 days	
2.3	Euthanasia and harvest of flaps for analysis as outlined in subtask 4 and SA3 subtask 6.3)	
2.4	Measurements of area of necrosis and analysis Blinded to xrt/sham and RTA/control	
3	**Administration of RTA 408, combined injury insult**	Luginbuhl
3.1	Order 40 Sprague Dawley rats.	
3.2	Weigh out RTA in aliquots	
3.3	Create 2X2 cm abdominal excisional wounds 2 hours prior to radiation/sham	
3.4	Injection of RTA or vehicle control on day of radiation through 3 days post radiation.	
3.5	*Biopsy collected at days 1,3,7,14,21,28 after combined injury*	
4	**Follow acute and chronic effects of combined injury**	Luginbuhl
4.1	Photodocumentation with measurement of wound contraction and healing	
4.2	Measurement of area of open wound (blinded to IR/sham and RTA/control.	
5	**Analysis of early injury**	Rodeck
5.1	Optomize antibodies for IHC	
5.2	Optimize qRT-PCR and mRNA protocols	
5.3	Biochemical characterization of tissue generated in 3.5 with antibodies, etc. optimized in 5.1 and 5.2	
6	**Analysis of late injury**	Rodeck
6.1	Optimize antiboides for IHC/mRNA (hydroxyproline, lysyl oxidase, TGF, collagens 1 and 3, transglutaminase2, etc.	
6.2	QC and optomization qRT-PCR protocols	
6.3	Beiochemical characterization of tissue generated in 2.3 wikth antibodies, etc. optimized in 6.1 and 6.2	
7	**Analysis of myelonmoncytic cells**	Rodeck
7.1	Characterize myelomonocytic cell infiltrates after irradiation and drug treatment	
8	**Analysis and conclusions**	Team